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Short communication

Reversed-phase high-performance liquid chromatographic analysis of atenolol enantiomers in rat hepatic microsome after chiral derivatization with 2,3,4,6-tetra-*O*-acetyl-β-D-glycopyranosyl isothiocyanate

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Abstract

A reversed-phase high-performance liquid chromatographic method for the determination of the enantiomers of atenolol in rat hepatic microsome has been developed. Racemic atenolol was extracted from alkalinized rat hepatic microsome by ethyl acetate. The organic layer was dried with anhydrous sodium sulfate and evaporated using a gentle stream of air. Atenolol racemic compound was derivatized with 2,3,4,6-tetra-*O*-acetyl-β-D-glycopyranosyl isothiocyanate at 35°C for 30 min to form diastereomers. After removal of excess solvent, the diastereomers were dissolved in phosphate buffer (pH 4.6)–acetonitrile (50:30). The diastereomers were separated on a Shimadzu CLC-C18 column (10 μm particle size, 10 cm×0.46 cm I.D.) with a mobile phase of phosphate buffer–methanol–acetonitrile (50:20:30, v/v) at a flow-rate of 0.5 ml/min. A UV–VIS detector was operated at 254 nm. For each enantiomer, the limit of detection was 0.055 μg/ml (signal-to-noise ratio 3) and the limit of quantification (signal-to-noise ratio 10) was 0.145 μg/ml (RSD <10%). In the range 0.145–20 μg/ml, intra-day coefficients of variation were 1.0–7.0% and inter-day coefficients of variation were 0.4–16.5% for each enantiomer. The assay was applied to determine the concentrations of atenolol enantiomers in rat hepatic microsome as a function of time after incubation of racemic atenolol.

Keywords: Enantiomer separation; Derivatization, LC; 2,3,4,6-Tetra-*O*-acetyl-β-D-glycopyranosyl isothiocyanate

1. Introduction

Atenolol, a β-adrenoceptor blocking agent, is used clinically for the treatment of angina pectoris and hypertension. Atenolol is usually marketed as a racemic mixture of two enantiomers. In general, like most β-adrenoceptor blocking agents, the β-adrenoceptor blocking activity appears to reside in the *S*-(−)-enantiomer [1]. Because enantiomers often differ in their metabolic fate, it is desirable to employ stereospecific analytical methodology to measure the concentrations of each enantiomer in samples of biological origin, such as rat hepatic microsomes containing atenolol racemate. Enantioselective HPLC bioassays for atenolol have been published, including chiral derivatizing agents (CDAs) [2–4], chiral stationary phases (CSPs) [5–8]

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and chiral mobile-phase additives (CMPAs) [9]. The matrices were biological fluids such as plasma, urine, etc. The chiral derivatizing agents used in the chiral separation of atenolol included (+)-1-(9-fluorenyl)ethyl chloroformate, (−)-menthyl chloroformate, and *S*-(−)-α-methylbenzyl isocyanate.

In order to study the in vitro metabolism characteristics of atenolol enantiomers, a stereoselective procedure to assay atenolol enantiomers in microsomes is needed. This report presents an analytical procedure for the individual enantiomers of atenolol in rat hepatic microsomes. The procedure involved extraction of atenolol from microsomes followed by derivatization at 35°C with 2,3,4,6-tetra-*O*-acetyl-β-D-glycopyranosyl isothiocyanate (GITC). The resultant diastereomers were then separated by reversed-phase high-performance liquid chromatography (HPLC) and detected by UV detection. The procedure was reproducible and sufficiently sensitive to allow for assay of the individual enantiomers in rat hepatic microsome for in vitro metabolism. Recently, the procedure was used to ascertain a stereoselective metabolism study of atenolol in rat hepatic microsomes.

2. Materials and methods

2.1. Materials

Atenolol was obtained from the National Institute for Drugs and Bioproducts Inspection (Beijing, China). 2,3,4,6-Tetra-*O*-acetyl-β-D-glycopyranosyl isothiocyanate (GITC), β-nicotinamide adenine dinucleotide phosphate (reduced form, β-NADPH), D-glucose 6-phosphate (G-6-P), and glucose-6-phosphate dehydrogenase (G-6-PDH) were all purchased from Sigma (St. Louis, MO, USA). Acetonitrile and methanol were HPLC grade, and sodium dihydrogen phosphate (NaH_2PO_4), sodium chloride (NaCl), concentrated ammonia water, sodium hydroxide, ethyl acetate and anhydrous sodium sulfate were all analytical grade.

Phosphate buffer, needed for the mobile phase and injection solutions, was prepared with sodium dihydrogen phosphate and adjusted to the desired pH (4.6) with concentrated phosphoric acid.

GITC solution, as the derivatization reagent, was prepared by dissolving GITC in acetonitrile to provide a concentration of 2.0 mg/ml.

Rat hepatic microsomes induced with dexamethasone sodium phosphate (Dex) were prepared as described by Gibson Skett [10] from rats (Sprague-Dawley, male, 160–230 g) which were treated with dexamethasone sodium phosphate (p.o. 100 mg/kg, 3 days). Microsomal protein was measured by the method of Lowry et al. [11] with bovine serum albumin as the protein standard.

2.2. Apparatus and chromatographic conditions

Chromatographic determination of the diastereomeric derivatives of atenolol was performed using a Shimadzu HPLC system which consisted of the following components: an LC-10 ATvp pump coupled to a manual injector with a 20 μl fixed loop, a Shimadzu CLC-C18 column (10 μm particle size, 10 cm×0.46 cm I.D.), and a SPD10Avp UV–VIS detector. The chromatographic data were collected and processed using an Upper Chromatopac Station Version 1.0 (Zhejiang University, Hangzhou, China).

The mobile phase consisted of phosphate buffer–methanol–acetonitrile (50:20:30). The column flow-rate was 0.5 ml/min with a guard column (10 mm× 5 mm I.D.; packed with YWG-$C_{18}H_{37}$, 10 μm particle size, Tianjin Second Chemical Reagent Industry, China). Eluted peaks were monitored at 254 nm and the atenolol concentrations were calculated by integration of the chromatographic peaks.

2.3. Standard solutions

Atenolol was accurately weighed, transferred to a volumetric flask and dissolved in water to make a 0.22 mg/ml standard solution. All standard solutions were stable for at least 2 months when stored at 4°C.

2.4. Calibration standard

To prepare calibration curves, standard samples of racemic compounds of atenolol were added to drug-free rat hepatic microsomes to give final enantiomer concentrations of 0.145, 0.5, 1, 2, 4, 10 and 20

μg/ml. Calibration curves were constructed by plotting peak area versus concentration.

2.5. Extraction and derivatization

Atenolol was extracted from 0.5 ml of the incubation mixture with 3 ml of ethyl acetate by vortexing for 2 min. After centrifugation at 1000 *g* for 5 min, 2.80 ml of the organic phase was transferred to a tube and dehydrated by adding about 70 mg anhydrous sodium sulfate. Then, 2.60 ml of the organic phase was transferred to another tube and evaporated to dryness using a gentle stream of air. The residue was reacted with 20 μl of GITC acetonitrile solution at 35°C for 30 min. After completion of the chiral derivatization, acetonitrile was evaporated by air flow and the residue was reconstituted with 300 μl of phosphate buffer–acetonitrile (50:30, v/v). Twenty microliters of the sample was injected into the HPLC system.

2.6. Metabolism study

Frozen microsomes were thawed to room temperature, and diluted with Tris–buffer (pH 7.4) to give a concentration of 2 mg protein/ml. The incubates contained 6.0 mg/ml G-6-P, 1.5 unit/ml G-6-PDH, and 3 μmol/l $MgCl_2$. The phase I metabolism was performed in 0.5 ml of the incubates bubbled with oxygen for 1 min in which there was 8 μg of atenolol racemic compound as the substrate. After pre-incubation at 37°C for 5 min, 10 μl of the NADPH solution (2.3 mg in 110 μl of 1% $NaHCO_3$) was added to the incubation mixture to start the reaction. The reactions were stopped by adding 0.2 g NaCl and 50 μl concentrated ammonia water at 0, 40, 80, 160, and 320 min. The mixtures were then analyzed as described in Section 2.5.

3. Results

3.1. Specificity

Fig. 1 shows the typical chromatograms obtained in our study. No interfering endogenous peaks were found for the drug-free microsome samples. In addition, there was no interference from reagents.

3.2. Extraction recovery

Recoveries of atenolol enantiomers were determined by comparing the peak area of an extracted sample containing a known amount of atenolol with the peak areas obtained from direct injections of a standard solution containing the same concentration of drug. The method recoveries for assay of atenolol enantiomers at concentrations from 1 to 20 μg/ml were 51.78±2.60% for *S*-(−)-atenolol and 49.91±2.71% for *R*-(+)-atenolol ($n = 4$).

3.3. Precision and accuracy

The precision and accuracy of the method were assessed by inter- ($n = 5$) and intra-assay ($n = 5$) validations at concentrations of 0.145, 0.5, 1, 2, 4, 10, and 20 μg/ml. Inter-assay variability was determined by replicate analyses of each of the spiked microsome samples on five separate days. The results (Table 1) showed a coefficient of variation (CV) of ≤20% and the relative errors for all concentrations were estimated.

3.4. Sensitivity

The limit of detection (signal-to-noise ratio 3) for each enantiomer was 0.055 μg/ml, and the limit of quantification, defined as the smallest sample concentration detectable (signal-to-noise ratio >10) in rat hepatic microsomes with adequate analytical precision (RSD <10%), was 0.145 μg/ml for each enantiomer.

3.5. Linearity

Drug-free microsomes were spiked with serial concentrations of atenolol racemate (0.145–20 μg/ml). Standard calibration curves were constructed by performing a linear regression analysis of the peak area (Y) of atenolol enantiomers versus their concentrations (X, μg/ml), i.e. *S*-(−)-atenolol, $Y = 883751.7X + 66363.6$, $r = 0.9998$; *R*-(+)-atenolol, $Y = 856150.9X + 92187.5$, $r = 0.9989$.

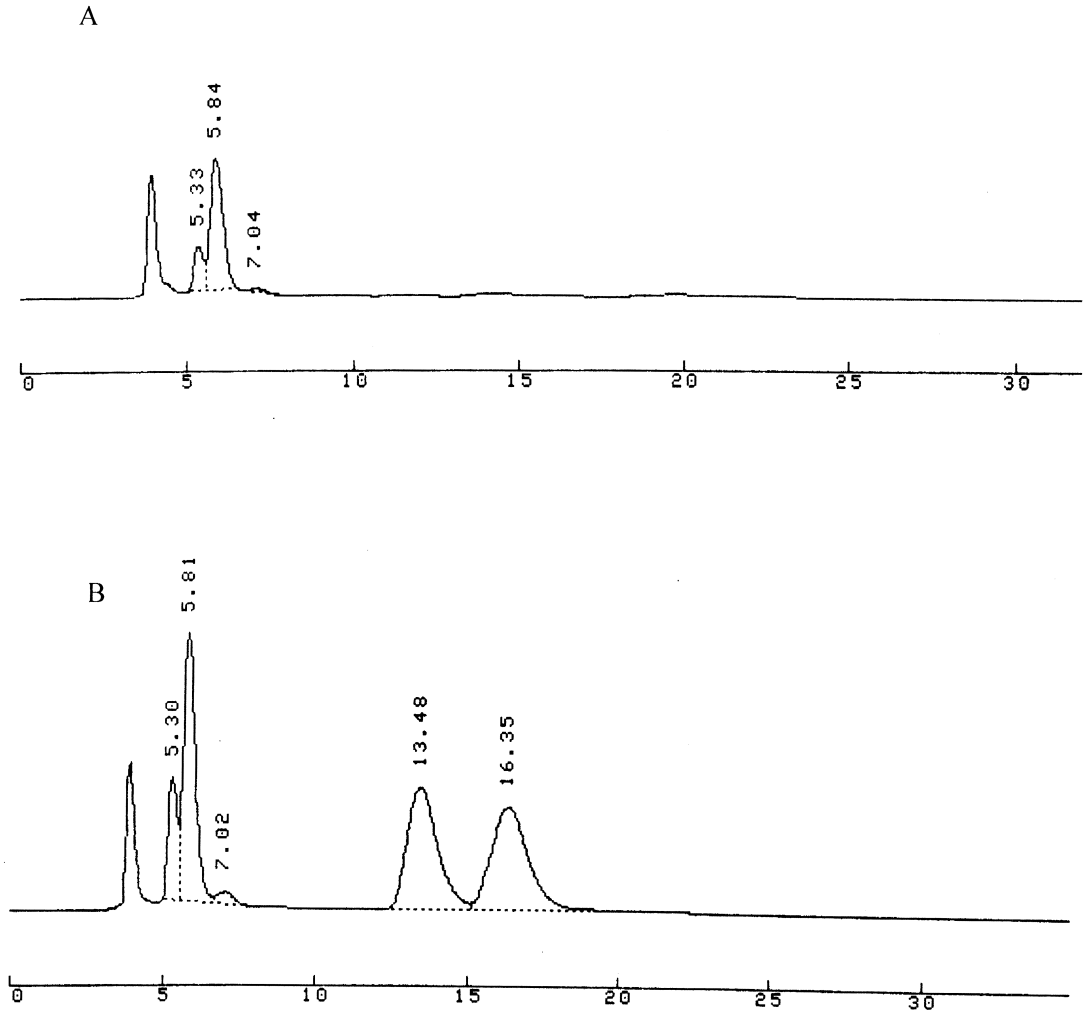

Fig. 1. Typical chromatograms of atenolol in rat hepatic microsome after chiral derivatization. (A) Blank rat hepatic microsome incubate. (B) Spiked rat hepatic microsome incubate. *S*-(−)-atenolol, $t_R = 13.64$; *R*-(+)-atenolol, $t_R = 16.35$.

4. Discussion

Enantioselective chromatography has been very useful and helpful for the study of stereoselective metabolism of enantiomeric drugs in vitro and in vivo [12–15]. This paper describes a simple, accurate, precise, and specific HPLC method for the determination of derivatives of atenolol enantiomers in solutions of rat hepatic microsome.

During the development of the enantioselective methodology, care was taken to optimize the reaction time for the conversion of isomers to their corresponding diastereomers in 20 μl GITC. Results showed that the formation of the diastereomers was nearly completed within 30 min after the addition of 20 μl GITC in acetonitrile (Fig. 2) and a linear calibration function was achieved over the whole calibration range. The influence of methanol, acetonitrile, and ethyl acetate on the derivatization reaction was investigated. It indicated that the derivatization reaction between atenolol and GITC should avoid residual water or methanol in the solvent. Selection of the solvent to dissolve the derivatives was made with acetonitrile, methanol and

Table 1
Intra- and inter-assay precision and accuracy at five concentrations

Added (μg/ml)	*n*	Found (μg/ml)					
		S-(−)-Atenolol	RSD (%)	Relative error (%)	*R*-(+)-Atenolol	RSD (%)	Relative error (%)
Intra-assay variability							
0.145	5	0.162±0.010	6.1	11.7	0.165±0.012	7.0	13.7
0.500	5	0.474±0.026	5.5	−5.2	0.473±0.028	6.0	−5.5
1.000	5	1.040±0.015	1.4	4.0	0.998±0.019	1.9	−0.2
2.000	5	1.983±0.038	1.9	−0.9	1.964±0.041	2.1	−1.8
4.000	5	3.800±0.099	2.6	−5.0	4.090±0.098	2.4	2.3
10.000	5	10.430±0.709	6.8	4.3	9.79±0.264	2.7	−2.1
20.000	5	19.150±0.192	1.0	−4.3	20.57±0.206	1.0	2.9
Inter-assay variability							
0.145	5	0.167±0.028	16.5	15.0	0.165±0.026	15.8	14.1
0.500	5	0.458±0.060	13.1	−8.5	0.462±0.061	13.3	−7.6
1.000	5	0.975±0.137	14.0	−2.5	0.966±0.131	14.0	−3.4
2.000	5	1.971±0.071	3.6	−1.5	1.980±0.041	2.1	−1.0
4.000	4	4.014±0.178	4.4	0.4	4.031±0.199	4.9	0.8
10.000	5	10.080±0.174	1.7	0.8	10.060±0.163	1.6	0.6
20.000	4	19.950±0.094	0.5	−0.3	19.96±0.083	0.4	−0.2

mobile phase. The derivatives were not dissolved completely and were unstable when methanol was present. Therefore, acetonitrile was selected as solvent for derivatization and phosphate buffer–acetonitrile (50:30, v/v) for dissolution of the derivatives prior to injection. The derivatives were stable for 3 h in phosphate buffer–acetonitrile (50:30, v/v).

Methanol–phosphate buffer was used as eluent for the efficient separation of diastereomeric derivatives, but was unable to separate the peak of the *R*-(+)-atenolol derivative from another peak identified as a possible degradation product. When acetonitrile–phosphate buffer was used as eluent, this peak was separated from that of *R*-(+)-atenolol, but the resolution of (*R*,*S*)-atenolol was not sufficient. Optimal resolution of the enantiomeric pair of (*R*,*S*)-atenolol was achieved with an eluent consisting of phosphate buffer–methanol–acetonitrile (50:20:30, v/v/v) (Fig. 3).

Water must be removed from the organic solvent when extracting atenolol from the microsomes. Alkaline medium is reasonable for the extraction of atenolol from incubates. Concentrated ammonia water was adopted as the alkaline reagent because it can be removed easily along with the organic solvent evaporated after extraction.

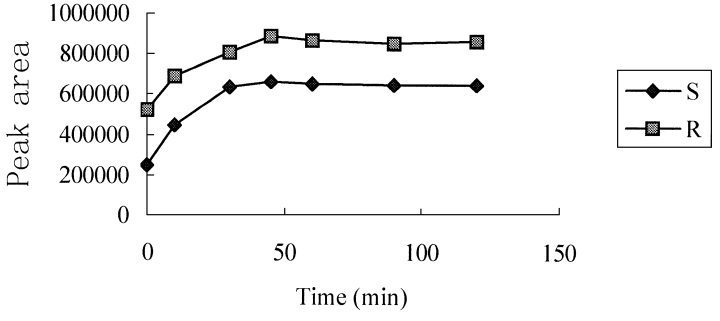

Fig. 2. Reaction times and the conversion of isomers of atenolol to their corresponding diastereomers.

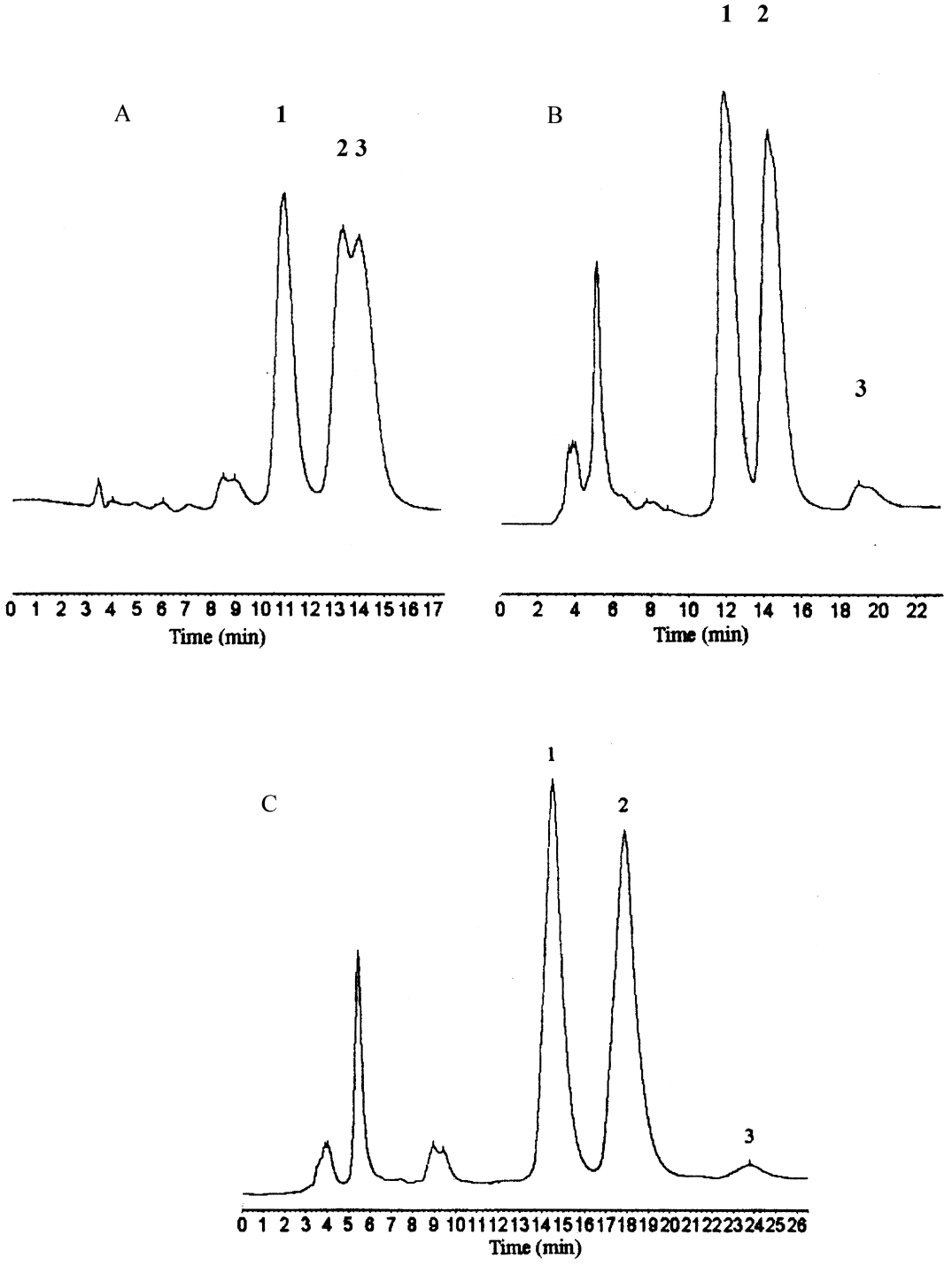

Fig. 3. HPLC chromatograms of atenolol derivatives eluted with different mobile phases. (A) Phosphate buffer–methanol (57:40); (B) phosphate buffer–acetonitrile (40:25); (C) phosphate buffer–methanol–acetonitrile (50:20:30). 1=*S*-(−)-Atenolol, 2=*R*-(+)-atenolol, 3=degradation product.

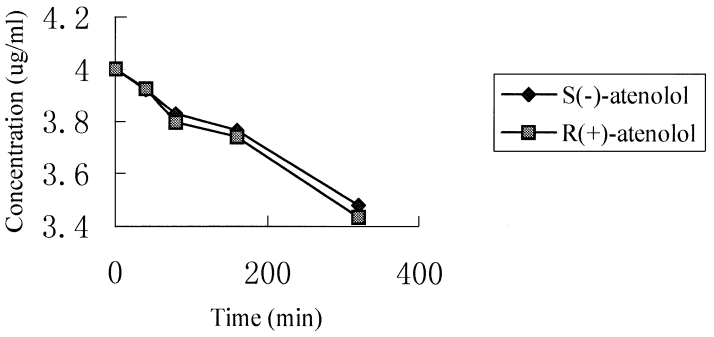

Fig. 4. Concentrations of atenolol in Dex-induced rat hepatic microsomes after different incubation times.

5. Application

The present method was devised to monitor atenolol concentration changes in rat hepatic microsomes for the study of in vitro drug metabolism. We tested the practical applicability of this procedure by assaying atenolol in microsomes induced by Dex after different incubation times. The concentrations of atenolol enantiomer in the microsome at the different incubation times are summarized in Fig. 4. Results showed that the amount of atenolol enantiomers was reduced to 13% within 320 min, and the enantiomers seemed to be metabolized at different rates. The stereoselectivity of metabolism should be studied with additional experiments.

The present method allows us to measure atenolol enantiomers adequately in enzymatic reactions and should be useful for further investigations of the enzyme kinetics of this drug in different enzymatic metabolic processes.

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